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(54) Title: EXPRESSION OF ACTIVE INTERFERON BETA 1 USING RECOMBINANT RNA REPLICONS

(57) Abstract

Compositions and methods are disclosed for producing recombinant human interferon beta–1. The compositions include recombinant RNA replicons containing a sequence encoding interferon beta–1. Methods utilizing the novel compositions consist of growing nonproducing cells to high cell densities and causing production of interferon beta–1 by the introduction of recombinant RNA replicons into the host cells.

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EXPRESSION OF ACTIVE INTERFERON BETA 1 USING RECOMBINANT RNA REPLICONS

1. INTRODUCTION

Compositions and methods are disclosed for producing recombinant human interferon beta 1. The compositions include recombinant RNA replicons containing a sequence encoding interferon beta 1. Methods utilizing the novel compositions consist of growing nonproducing cells to high cell densities and causing production of interferon beta 1 by the introduction of recombinant RNA replicons into the host cells.

2. BACKGROUND OF THE INVENTION

Human interferon beta-1 is a glycosylated protein which is secreted by fibroblasts in response to viral infection or exposure to double-stranded RNA. Interferon beta has antiproliferative activity and has been used for the chemotherapy of certain types of tumors. Its clinical importance has increased since the discovery that interferon beta-1 can retard the progression of certain forms of multiple sclerosis. Interferon beta-1 has been manufactured in several different ways. Recombinant interferon beta-1 has been produced by inserting the isolated, purified DNA sequence encoding the interferon beta-1 protein into a DNA expression vector following a functional transcription initiation sequence and introducing such a DNA expression vector into a bacterial host or a mammalian cell line. In contrast to the bacterial expression of interferon beta-1, which yields an unglycosylated interferon product, the mammalian expression product is glycosylated and, thus, has greater similarity to the human form of interferon beta-1. Although both glycosylated and unglycosylated interferon beta-1 are active in the human organism, first clinical data indicate that a glycosylated form of interferon beta-1 displays less side effects and can be applied in a more convenient regimen of administration.

Although a glycosylated interferon beta-1 product has several clinical advantages over the unglycosylated form, its production in recombinant mammalian cells is highly complicated. In addition to the general difficulties of a mammalian cell culture process, the growth-inhibitory effect of interferons interfere strongly with the requirements of an efficient cell culture process (Stanley, L. et al. Science 233, 356, 1986, Sokawa et al. Nature, 268, 236, 1977, Balkwil, F. and Taylor, Papadimitriou, J. ibid., 269, 798, 1978, Lundgren, E. et al. J. Gen. Virol. 42, 589, 1979, Creasey, A. A. et al. Proc. Natl. Acad. Sci. USA, 77, 1471, 1980, Tamm, I. in Mechanism of Interferon Action. L. M. Pfeiffer, Ed. CRC Press, Boca Raton, Florida, 1986). Difficulties during the selection of producing clones were reported. In particular, clones producing high levels of interferon beta grew slowly, and only a few clones that grew out of this "crisis" could be used for production (Innis, M. and McCormick, F. in Methods in Enzymology vol. 119 [57] 397, 1986).

In an ideal cell culture process, a high cell yield is achieved in a first phase of fast growth, which is followed by a second phase of efficient product formation. Premature synthesis of interferon beta-1 induces a growth arrest at low cell density. It is therefore essential that the expression of interferon beta-1 be regulated. The two important requirements of fast growth to high cell density and high productivity contradict each other, as long as the two phases cannot be separated strictly. Consequently, clones that exhibit high expression will show poor growth characteristis and vice versa. Although promoters exist by which the expression can be induced by external chemical or physical parameters, the baseline expression of such promoters is frequently too high to eliminate growth restriction by an inhibitory protein product such as interferon beta-1. Moreover, i) the site of intergration in the genome, ii) the position of the promoter relative to a transactivator and iii) the copy number inserted in the genome are all parameters which affect the levels of expression in the induced and uninduced state. Since none of these parameters can be controlled, and since nothing is known about their ideal configuration, the only way to adress this problem remains the careful investigation of background

expression, induced expression and growth characteristics of tens or hundreds of individual clones. A clone selcted in this laborous way will be a compromise of modest growth characteristics and productivity. It has not been possible so far to identify tight promoters with high inducibility that are not dependent on the factors mentioned above.

A chosen clone must then be characterized based on small-scale process evaluation of growth characteristics and interferon production. Due to the genetic instability of transformed cells such as Chinese Hamster Ovary (CHO) or Baby Hamster Kidney (BHK) cells, recombinant clones derived from such cell lines will exhibit changed growth or productivity over time. The long-term stability of a clone, which is a critical requirement for use in manufacturing, can only be addressed by careful investigation of many different clones in lengthy experiments. The master cell bank so derived must be constantly checked for possible changes in the stored cells which compromise the reproducibility of the production process and the quality of the resulting product.

Ideally, cells used for the manufacture of a growth inhibitory or toxic product would be grown to high cell density without any contact or interference with the heterologous polynucleic acids coding for such a product. Subsequently, protein biosynthesis and processing capacities of the host are switched by the introduction of polynucleic acids which direct the production of such product. The invention disclosed herein fulfils these criteria.

By replacing the structural protein-encoding sequences with heterologous sequences in the genome of alpha viruses, recombinant RNA replicons have been constructed recently that can be used for heterologous protein expression (Xiong, C. et al. Science 243, 1188 (1989)). The recombinant RNA molecules are introduced into host cells, and, due to their ability to replicate and amplify, will provide sufficient template for translation of the heterologous protein over an extended period of time. These replicons can be packaged in the presence of wild-type virus. A significant improvement was made with the development of defective helper RNAs (Bredenbeek, P. J. et al. J. Virology, 67, 6439 1993). These RNAs contain the *cis* acting

sequences required for replication as well as the subgenomic RNA promoter which drives the expression of the structural protein genes. In cells cotransfected with both the replicon and the defective helper RNA, viral nonstructural proteins translated from the recombinant RNA allow replication and amplification of the defective helper RNAs to produce the virion structural proteins. Since the helper RNAs lack packaging signals, they are not packaged and, thus, the virion particles produced in this way undergo only one round of infection and are not pathogenic. These virion particles can be used to infect a culture of host cells simply by addition to the culture liquid. The recombinant RNA replicon enters the cell and redirects cellular functions to the production of the viral and heterologous proteins. Alpha virus-derived vectors have been used to express a small number of proteins such as bacterial chloramphenicol transferase, beta galactosidase or tissue plasminogen activator in a variety of animal cells (Bredenbeek et al. J. Virology, 67, 6439, 1993, Hahn, C. S. et al., Proc. Natl. Acad. Sci. USA, 89, 2679, 1992, Huang H. V. et al. US Patent 5,217,879, Johanning et al. Nucleic Acid Res. 23, 1495, 1995). To date glycosylation of any heterologous protein expressed using alpha virus-derived vectors has not been characterized.

It is not obvious from the prior art that production of a heterologous glycoprotein with sufficient activity and suitable glycosylation can be achieved with recombinant RNA replicon-based technology because initiation of the replication cycle has been shown (a) to rapidly stop synthesis of all host cell proteins (Strauss, J. H. and Strauss, E. G. Microbiol. Rev. 58, 491, 1994) and (b) to result in lysis of the infected cells after about 24 hours (Schlesinger, R. W. in The Togaviruses, Academic Press, New York, 1980). Thus, entry of the replicon into the host cell begins immediately to interfere with and eventually to terminate normal host cell functions. Among the cellular functions that must be affected at some time after entry of the RNA replicon are the ones which catalyse processing and secretion of proteins. It is not obvious that a host cell subjected to this insult can process, glycosylate, and secrete a recombinant RNA replicon-encoded heterologous protein with enough efficiency to provide a

significant quantity of reasonably glycosylated heterologous protein product. To date no reports are available that would teach one skilled in the art that recombinant RNA replicons can be used to produce active interferon beta-1.

Surprisingly, the inventors found compositions and methods that enable the production of active interferon beta-1 using RNA replicons. The in this manner produced interferon beta-1 shows *in vitro* activity and activates an anti viral state in Hep2 cells.

To date no mammalian cell culture processes used to produce protein products in industry, including any for effective production of active interferon beta-1, have been reported which do not employ animal serum additive to the culture or cell storage medium at some stage(s) of the process. Animal serum is a conventional supplement to mammalian cell culture media. Because serum is an undefined mixture containing hundreds of different proteins, there is no scientific basis for identifying the precise functions which serum serves in cell culture. Suggested functions of serum include (i) provision of growth factors which stimulate cell proliferation, (ii) provision of nutrients missing from the basal medium, and (iii) buffering of the culture against inhibitory metabolites or proteins produced by the cultured cells. Adding animal serum to cell culture used for manufacturing of pharmaceutical products is extremely undesirable from several viewpoints, including high variability in the functional effects of different lots of serum, possible contamination of serum by pathogens, interference of serum proteins with recovery and purification of the heterologous protein product, and high cost. The most desirable cell culture medium for practical use in pharmaceutical manufacturing is one which is chemically defined and which contains no protein (except those proteins which the cells may secrete or otherwise release into the medium during the cultivation).

To date there are no reports of heterologous protein expression using recombinant RNA replicons in a protein-free mammalian cell culture process. It is not obvious that reasonable production of a heterologous protein can be achieved in serum-free culture using a recombinant RNA replicon vector since serum may provide factors important to the cells for effective entry

and replication of the recombinant RNA replicon and expression of the heterologous protein. Compositions and methods are provided herein that allow the production of active interferon beta-1 in serum-free and in protein-free media.

3. SUMMARY OF THE INVENTION

The present invention is directed to an improved process for synthesis of active interferon beta-1 by mammalian cells in culture. The process is based upon stable, easily stored cells and recombinant RNA replicons. The requirement of introduction and cloning of foreign DNA into the host mammalian cells is avoided entirely, making possible use of well-characterized mammalian cell hosts which have been optimized for growth and production in culture. In conducting the process, these cells grow efficiently without any contact or interference with heterologous polynucleic acids. Subsequently, protein biosynthesis and processing capacities of the host may be switched, at an optimum point in the process, to production of interferon beta-1. The overall process produces high levels of active interferon beta-1.

The invention encompasses uses of recombinant RNA replicons to produce active interferon beta-1 in mammalian cell culture and methods of interferon beta-1 production in mammalian cell culture directed by recombinant RNA replicons. Preferably replicons derived from the alpha virus subgroup and more preferably of the Sindbis virus species are used. In one of its embodiments recombinant RNA replicons are introduced into the mammalian cell by transfection, electroporation, or lipofection and the like. In another embodiment the recombinant RNA replicons are packaged into virion particles, which are subsequently used to infect host cells.

In another embodiment of the invention the entire cell culture and interferon beta-1 production process is conducted in serum-free medium, avoiding any intermediate cell-medium separation and medium replacement, and providing a final cell

culture broth from which active interferon beta-1 can be recovered.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: The amino acid sequence of human interferon beta-1 is shown.

Figure 2: The nucleotide of human interferon beta-1 is shown (HincII fragment).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to replicating RNA molecules (RNA replicons) containing a sequence encoding interferon beta-1 and methods for their utilization for the production of biologically active interferon beta-1.

In the context of this disclosure the following terms shall be defined as follows unless otherwise stated.

"biologically active interferon beta-1": a glycoprotein with the amino acid sequence shown in figure 1 and with the property of activating an antiviral state in Hep2 cells as determined by the cytopathic effect reduction (CPER) assay (Maeger, A., in: Lymphokines and Interferon. A practical approach, Ed. M.J. Clemens, A. G. Morris, A. J. H. Gearing, IRL Press Oxford, Chapter 9, 129-147, 1987).

"replication" of RNA molecules means production of full length RNA equivalents of plus polarity.

"replicon" means an RNA molecule, which upon introduction into a host cell, is replicated.

"recombinant RNA replicon" means a recombimbinant RNA molecule containing an RNA sequence coding for a product protein, which, upon introduction into a host cell, is replicated and at least a portion of it is amplified and translated to give the protein product.

"amplification" of an RNA molecule means production of a plus polarity RNA molecule using a minus strand nucleic acid molecule as a template.

"transfection" is understood to include any means, such as, but not limited to, adsorption, microinjection, electroporation, lipofection and the like for introducing an exogenous nucleic acid molecule into a host cell.

"infection" means the introduction into a host cell of nucleic acid molecules which are packaged in a virus or virion particle.

The compositions and methods disclosed are based upon use of recombinant RNA replicons to direct production, within a cultured mass of mammalian cells, of large quantities of RNA which is translated to produce large quantities of interferon beta-1 polypeptide. Surprisingly, by timing carefully the time of introduction of the recombinant RNA replicon into the host cells, and the interval after introduction during which interferon beta-1 accumulated in the culture medium is harvested, large quantities of reasonably glycosylated, sialylated and reasonably homogeneous active interferon beta-1 can be produced.

Accordingly, the RNA replicons suitable for use in this process are those which have been engineered to enable operative fusion of nucleic acid coding for interferon beta-1 to the sequences necessary for replication of the RNA molecule. In particular the replacement of the RNA sequences encoding the structural proteins of an alpha virus with an interferon beta-1-encoding RNA sequence yields a construct with the ability to replicate and to direct the host to synthesize interferon beta-1 protein. Such vectors include, but are not limited to, the Sindbis virus vectors (e.g., pSinRep5, Bredenbeek, P. et al. J. Virology 67, 6439, 1993), and the Semliki forest virus vectors (e.g., pSFV, Trends Biotechnol. 11, 18, 1993).

Beginning with a purified preparation of a suitable interferon beta-1-encoding replicon, such recombinant RNA molecules can be introduced into mammalian cells by techniques such as electroporation or transfection or, alternatively, can be packaged into viral particles with the aid of helper viruses.

The invention also encompasses expression of degenerate variants of RNA sequences which encode the amino acid sequence of the interferon beta-1 protein, interferon beta-1 mutants, and functional equivalents of interferon beta-1 encoded by nucleotide sequences which hybridize to the complement of the nucleotide sequence encoding interferon beta-1. For example, the nucleotide sequence may be altered so as to optimize amino acid codon usage for expression in the chosen host cell.

A variety of host cells, preferably mammalian cells, can be used to produce interferon beta-1 using this invention. CHO cells are particularly suitable for this process because they have the potential to glycosylate heterologous proteins and in particular interferon beta-1 in a manner relatively similar to human cells (Kagawa, Y. et al. J. Biol. Chem. 263, 17508, 1988) and because they can be selected (Zang, M. et al. Bio/Technology, 13, 389, 1995) or genetically engineered (Renner W. A. et al. Biotech. Bioeng. 47, 476, 1995, Lee K. H. et al. Biotech. Bioeng. 50, 336, 1996) to grow in serum-free medium and to grow in suspension.

A number of different bioprocess parameters can be varied in order to increase the amount of active interferon beta-1 which is produced in a recombinant RNA replicon-based cell culture process. The conditions under which the host cells are grown prior to exposure to the viral vector (such as medium composition, pH, oxygen concentration, agitation, and, for the case of anchorage-dependent cells, the surface provided and the carrier of that surface) influence both the cell number density achieved at a given time, and also the physiological state of those cells and hence their expected response to exposure to the recombinant RNA replicon. The time at which the recombinant RNA replicon is added to the previously unexposed cell culture is a critical parameter to achieve good results with this process, as is the concentration at this time of recombinant RNA replicon relative to the concentration of viable cells, which is determined by the amount of recombinant RNA replicon added. Subsequent to addition of the recombinant RNA replicon to the process, and depending on the host cell line used and prior process conditions, all of the cell culture process conditions

mentioned earlier can be chosen to achieve high productivity of active interferon beta-1.

The overall cell culture process employing recombinant RNA replicons for production of active interferon beta-1 of this invention can be implemented in a variety of bioreactor configurations (for example, but not limited to, stirred-tank, perfused, membrane enclosed, encapsulated cell, fluidized bed, and air-lift reactors) and scales (from laboratory T-flasks to hundreds or thousands of litres), chosen to accommodate the requirements of the host cell line utilized (such as anchorage-dependent growth), to achieve the most active interferon beta-1 production, and to facilitate subsequent recovery and purification of active interferon beta-1.

The invention is illustrated by examples of active interferon beta-1 expression in Baby Hamster Kidney and Chinese Hamster Ovary (CHO) cells. An RNA sequence encoding interferon beta-1 protein is inserted into the Sindbis virus-based expression vector pSinRep5. Electroporation of pSinRep5-IFN RNA into BHK cells results in subsequent production of active interferon beta-1. When coelectroporated together with DH-BB RNA (directing the expression of the virus structural proteins), infectious particles are produced which can be used for subsequent infection of another cell line; e.g., CHO cells. The supernatant of a CHO cell culture infected with these particles is tested for interferon beta-1 activity. Surprisingly, high levels of active, glycosylated and sialylated interferon beta-1 can be recovered from these cultures.

Thus, the present invention is applicable for efficient production of active interferon beta-1 in a class of unusually flexible, easily optimized processes. The method can be implemented using different host cells, different recombinant RNA replicons, and bioreactor hardware and operating protocols chosen to maximize production of the preferred glycoforms of active interferon beta-1 for each cell line-vector combination.

5.1 CONSTRUCTION OF RECOMBINANT RNA REPLICONS FOR INTERFERON BETA-1 EXPRESSION

This invention embodies use of an RNA sequence encoding interferon beta-1 protein, with appropriate RNA-encoded signals for propagating this sequence, and its introduction into cultured cells such that the cells subsequently replicate this interferon beta-1-coding RNA sequence, amplify it and translate it to produce active interferon beta-1. The efficient utilization of recombinant RNA replicons of the present invention minimally requires that the recombinant RNA molecule can be replicated (e.g., produce a minus strand from which replicates of the recombinant RNA molecules with plus polarity are made) in the host cell, thereby increasing the number of interferon beta-1 coding sequences. Preferably a desired portion of the recombinant RNA molecule can be amplified.

In one preferred embodiment the structural protein-encoding sequences of an alpha virus are replaced with a coding sequence for human interferon beta-1. When inserted downstream from the start of the alpha virus subgenomic RNA, the template is amplified and large quantities of interferon beta-1-encoding RNA are produced. Such recombinant RNAs are self-replicating and can be introduced into cells as naked RNA, but they require *trans* complementation to be packaged and released from cells as infectious virion particles.

In a particularly preferred embodiment high levels of interferon beta-1 expression can be achieved using an engineered form of the Sindbis virus-based expression vector designated pSinRep5 (Bredenbeek, P. et al. J. Virology 67, 6439, 1993), to obtain a recombinant Sindbis virus-based vector (designated pSinRep5-IFN) which directs expression of active interferon beta-1 in mammalian cells. Cotransfection of the recombinant RNA replicon with a helper RNA providing the structural proteins required for packaging of the recombinant replicon leads to the release of infectious particles. Useful helper RNAs include, but are not restricted to, the helper RNAs designated DH-BB and DH-EB and DI(26S).

In the same way as safe RNA replicons were constructed by *trans* -complementation of the structural proteins with helper virions, it is also possible to substitute functions encoded on the replicon by an RNA polymerase II-dependent expression cassette inserted in the nucleus of the host cell. It was shown for example that coexpression of the Sindbis nonstructural proteins P123 and P34 establishes a functional RNA replication and transcription complex (Lemm, J. A. J. Virology, 67, 1905 (1993)). In the same manner the sequence coding for P4 can be deleted from the replicon and the sequence coding for P34 can be incorporated in an RNA polymerase II expression cassette into the nucleus of the host cell. Of course other functional replicon-host systems can be constructed by *trans*-complementation of other functions encoded by viral RNA sequences. Another way to improve the safety of recombinant RNA replicons is the use of temperature sensitive mutations which allow replication of the replicon only at the permissive temperature (Burge, B. W. et al. Virology, 30, 204 (1966), Strauss, J. H. et al. in The Togaviruses, R. W. Schlesinger, Ed. (Academic press, New York, 1980), Keranen, S. et al. J. Virology, 32, 19 (1979), Barton, D. J. et al, J. Virology 62, 3597 (1988)).

Several other RNA viruses can be modified in functionally similar ways to obtain recombinant RNA replicons suitable for use as expression vectors. Semliki forest virus has basically the same genomic organization as Sindbis virus. It is clear that the same principles of the present invention can be applied to other RNA replicons; e.g., these derived from the Semliki forest virus genome, without leaving the scope of the invention.

Based on the present recombinant RNA replicon technology, the general principles by which other recombinant RNA replicons can be developed, as new RNA viruses are discovered and characterized, are clear. The present invention embodies use of all such vectors, engineered to contain interferon beta-1-encoding RNA, to synthesize active interferon beta-1 in a cell culture process.

5.2 CHINESE HAMSTER OVARY (CHO) AND OTHER CULTURED MAMMALIAN CELL HOSTS

One major advantage of the present invention is the ability to use one recombinant RNA replicon engineered to express interferon beta-1 with any of a large variety of cell hosts. It is well known that, for example, Sindbis virus has a wide host range. In the laboratory it infects cultured mammalian, reptilian, amphibian, and some insect cells (Clark, H. F. J. Natl. Cancer Inst. 51, 645 (1973), Leake, C. J. J. Gen. Virol. 35, 335, (1977), Stollar, V. in The Togaviruses, R.W. Schlesinger, Ed. Academic press, New York, (1980), pp.583-621). Alternative embodiments of the present invention use host cells which can be maintained or propagated in culture, and especially those which have been demonstrated to have the capacity to express heterologous glycoproteins. Mammalian cell lines which fulfil these criteria include, but are not limited to, CHO, BHK, Vero, HeLa, MDCK and the like.

A preferred embodiment of the present invention employs one of several alternative cell lines derived from CHO cells. Different CHO cell lines offer different process characteristics, and may provide different glycosylation of heterologous proteins expressed therein. The wild-type CHO cell line designated K1 requires serum for growth in culture and grows attached to a surface. By long-term culture under conditions restricting serum access or selecting for suspension growth, mutant CHO cell lines able to grow in serum-free medium and/or in suspension can be isolated (Zang, M. et al. Bio/Technology, 13, 389, 1995). By genetic modification of CHO K1 cells, a modified cell line designated CHO K1:cycE was obtained which grows as suspended single cells in protein-free medium (Renner W. A. et al. Biotech. Bioeng. 47, 476, 1995,). CHO mutants, such as those designated LEC10, produce glycoproteins with different glycosylation than parental CHO cells (Stanley, P. Glycobiology, 2, 99, (1992)). Alternatively, CHO cells may be genetically modified to alter activities of oligosaccharide biosynthesis enzymes, with the resulting capability to synthesize glycoproteins with

correspondingly modified oligosaccharides (Minch et al. Biotechnol. Prog. 11, 348, 1995). By choosing among these alternative embodiments, this invention provides alternative process routes for production of active interferon beta-1 which differ in process characteristics and in the nature of the product.

5.3 MAMMALIAN CELL BIOREACTOR DESIGN AND OPERATION

The mammalian cells suitable for use in this invention can be maintained or propagated under a variety of conditions. For example, CHO-based cell lines have been cultivated in many different cell culture media including, but not limited to, Ham's F12, DMEM-F12 (1/1), and FMX-8 alone or supplemented with fetal calf serum, insulin, or basic fibroblast growth factor. Furthermore, this invention embodies the use of a range of cultivation conditions, both before and after addition of recombinant RNA replicon to the process. For example, CHO cells have been cultured under a range of medium pH values, temperatures, CO_2 and O_2 concentrations, and concentrations of added proteins or animal serum (Beuvery, E. C. et al. in Animal Cell Technology, Developments towards the 21 st Century, Kluver Academic Publishers, Dordrecht, 1995). These provide numerous ways within the scope of this invention to manipulate the amount of the active interferon beta-1 produced and the nature of this interferon beta-1 by changing glycosylation.

Depending on the requirements and responses of the chosen host cell line, several different types of reactors can be utilized for the cell culture process of this invention. These include, but are not limited to, roller bottles, T-flasks, and spinner flasks, which provide relatively little opportunity for monitoring and control of culture conditions during operation, and stirred tanks, air-lift reactors, fluidized bed reactors, and hollow-fiber and other membrane reactors (Bailey, J. E. and Ollis, D. F. Biochemical Engineering Fundamentals, Second Ed. McGraw Hill, New York, 1986). For anchorage-dependent cell lines, the available surface area, and hence the maximum cell capacity,

of the reactor can be increased by use of microcarriers and other macroporous materials and devices. The surfaces of these materials can be modified physicochemically, or by attachment of peptides or other biochemicals, to maximize the cell density and production activity of the culture.

This invention encompasses use of special procedures for adding and/or removing medium during the cell culture process to maximize the production of active interferon beta-1 in a cell culture process using recombinant RNA replicons. Because this recombinant RNA replicon technology provides exceptionally clear delineation of an initial cell growth phase of host cells only, and a subsequent production phase after addition of recombinant RNA replicons, the addition of supplemental nutrients and removal of medium containing metabolic products (especially in the growth phase of the process) and of active interferon beta-1 (only in the second, production phase of the process) can be done at times and at rates chosen to maximize the efficiency and productivity of the overall process, and to facilitate downstream purification.

6. EXAMPLES

Example 1: Synthesis of an interferon beta-1-encoding recombinant RNA replicon

The synthesis of the Sindbis expression vector pSinRep5 is described in Bredenbeek et al. J. Virology, 67, 6439 (1993); this vector can be purchased from Invitrogen (San Diego, CA). cDNA coding for interferon beta-1 can be cloned with the standard techniques. A 767 bp Hinc II fragment is subcloned into the Eco RV site of pBluescript KS-. A clone with insert in correct orientation is identified and an Xba 1, Apa 1 fragment is isolated and ligated into the Xba 1, Apa 1 sites of the Sindbis virus-based expression vector pSinRep5. The ligation product is then transformed into the E. coli strain DH5 alpha (Sambrook, J. et al. Molecular cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press. Plainview, New York, 1989).

A clone with insert in the correct orientation is identified and sequenced to confirm the presence and the correct sequence of interferon beta-1 DNA. Sufficient amounts of the expression plasmid are produced and purified using CsCl gradient centrifugation (Sambrook et al. ibid.)

Prior to *in vitro* transcription 5 micrograms of the template pSinRep5-IFN are linearized with the restriction enzyme Not I. The reactions are terminated with EDTA at a final concentration of 25 mM. After phenol extraction the DNA is ethanol precipitated. In order to avoid degradation of the RNA by RNases all steps are carried out with and in plasticware that has been previously treated with DEPC. Also all solutions are DEPC treated.

In vitro transcription is carried out as described by the manufacturer of the InvitroScript Cap Kit (Invitrogen, San Diego, CA). One microgram of pSinRep5-IFN is used for *in vitro* transcription reactions to yield about 20 micrograms of RNA as estimated on a nondenaturing agarose gel.

The recombinant RNA molecules produced in this way act as replicons. Recombinant RNA replicon is electroporated into BHK cells as follows: BHK 21 cells grown in T150 flasks to 90 % confluency in complete MEM alpha medium supplemented with 10% FCS are detached by trypsinization. To inactivate the trypsin the cells are taken into 5 ml complete MEM alpha. The cells are then washed once with serum-free MEM-alpha medium and then twice with RNase-free PBS without cations (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4, DEPC treated). After the two washes the cells are taken up in RNase-free PBS without cations at a concentration of 10^7 cells/ml. 0.5 ml of this cell suspension is placed into a 0.4 cm electroporation cuvette. For electroporation approximately 10 micrograms of capped pSinRep5 RNA are added to the cell suspension and mixed thoroughly. The cell suspension is pulsed twice at 2125 V/cm with a capacitance of 50 µF. The electroporated cells are placed for 5 minutes on ice for recovery and then transferred to 9.5 ml of complete MEM alpha medium. The cells are plated in a T75 flask and, 7 hours postelectroporation, dactinomycin (1 µg/ml) and [^{3}H]uridine (20 µCi/ml) are added. 4 hours later cellular RNA is isolated with the aid of the RNA

Purification Kit (Pharmacia, Piscataway, N.J.) and analyzed by electrophoresis on an agarose gel. RNA bands are visualized by fluorography. Two major strong bands are visible on the gel. The larger band corresponds to the replicated recombinant vector RNA and the smaller to the subgenomic RNA containing the interferon beta-1 encoding sequence.

Example 2: Production of active interferon beta-1 by introduction of an interferon beta-1-encoding recombinant RNA replicon via electroporation.

The interferon beta-1 encoding RNA replicon is introduced into BHK cells by electroporation as described in example 1. 36 hours postelectroporation interferon beta-1 activity in the supernatant is assayed by the cytopathic effect reduction assay as described using Hep2 cells and encephalomyocarditis virus (EMCV). In the unelectroporated sample and in the beta galactosidase-encoding sample, only baseline levels of interferon activity can be measured. The supernatant of the sample electroporated with the interferon beta-1-encoding RNA replicon gives rise to cytopathic effect reduction.

Example 3: Production of active interferon beta-1 in CHO cells by infection with an interferon beta-1-encoding recombinant RNA replicon packaged in virion particles.

Pseudovirion particles are made by coelectroporation of the recombinant RNA replicon pSinRep5-IFN with the helper RNA DH-BB into BHK cells. 10 micrograms of each RNA are simultaneously electroporated as described in example 1. The electroporated cells are transferred into 10 ml of complete MEM alpha medium in a T75 flask. 30 hours postelectroporation the supernatant which contains the pseudovirions is collected. Cell debris is removed by centrifugation for 10 minutes at 2,000 g. The virion-containing supernatant can be frozen in an ethanol / dry ice bath at -80°C. As a control beta galactosidase-encoding pseudovirions are produced according to the same method.

CHO K1 cells in separate cultures are infected with the pseudovirions encoding recombinant interferon beta-1 and beta galactosidase. CHO K1 cells are grown to 70% confluency in FMX-8 medium (Zang, M. et al. Bio/Technology, 13, 389, 1995) supplemented with 10 % FCS in a T75 flask. 250 µl of the virus stock solution are mixed with 500 µl of FMX-8 medium supplemented with 1% FCS and added to the T75 flask. After one hour incubation in the CO_2 incubator at 37°C on a rocking plate, 10 ml of FMX-8 +10% FCS are added and incubation is continued for 24 hours. The supernatant is collected and cell debris is removed by centrifugation for 10 minutes at 2,000 g. Assaying the supernatants prepared in this manner according to example 1 results in a cytopathic effect reduction in the sample infected with the interferon beta-1-encoding replicon.

Example 4: Production of sialylated interferon beta-1 using an interferon beta-1-encoding recombinant RNA replicon.

The supernatants of the experiments of Example 2 and 3 are subjected to an ELISA assay for the determination of neuraminic acid residues bound to interferon beta-1. Anti-interferon beta-1 antibodies are immobilized on a microtiter plate. Samples from the cultures expressing interferon beta-1 and beta galactosidase are applied to the microtiter plate. For neuraminidase treatment 1 mU of neuraminidase (Vibrio cholerae, Sigma) is added and incubated for 2 h at 37°C on a rocking plate. 30 microgram/ml of digoxygenin-labelled MAA lectin is added to the plate after three washing steps with TBS. After washing, sheep anti-digoxigenin Fab fragments at a dilution of 1/500 are added to each well and incubated for one hour at room temperature. The plates are washed six times with TBS and 100 µl freshly prepared substrate solution is added to the wells. Depending on its intensity the reaction is stopped after 2-5 minutes by the addition of 50 µl of 2 molar sulfuric acid. Absorbance is measured at 450 nm against 490 nm wavelength on a ELISA reader. The samples containing the expression products of beta galactosidase are not distinguishable from a baseline that was determined by using uninfected CHO cell

culture supernatant. The sample from interferon beta-1-encoding replicons has a significantly increased OD whereas this sample, when pretreated with neuraminidase, shows only baseline levels.

Claims

1. A recombinant RNA molecule, containing an RNA sequence coding for human interferon beta-1, or a sequence hybridizing under stringent conditions to the complementary strand of a sequence coding for interferon beta-1, which, upon introduction into a host cell, is replicated and at least a portion of it is amplified and translated to give human interferon beta-1 protein.

2. The recombinant RNA molecule of claim 1 wherein the sequences required for replication and amplification are derived from viral RNA sequences.

3. The recombinant RNA molecule of claim 2 wherein the viral RNA sequences are derived from an alpha virus genome.

4. The recombinant RNA molecule of claim 3 wherein a coding sequence for human interferon beta-1 is located downstream from the start of the alpha virus subgenomic RNA sequence.

5. The recombinant RNA molecule of claim 4 wherein the alpha viral sequences are sequences derived from the Sindbis virus genome.

6. The recombinant RNA molecule of claim 4 wherein the alpha viral sequences are sequences derived from the Semliki forest virus genome.

7. The recombinant RNA molecule of claim 5 wherein the RNA molecule is pSinRep5-IFN.

8. A method for synthesizing interferon beta-1 comprising the steps of growing host cells under suitable culture conditions to a sufficient cell density, introducing RNA molecules of claims 1-7 into the cells and collecting interferon beta-1.

9. The method of claim 8 wherein the RNA molecule is packaged into a viral particle prior to introduction into the host cell.

10. The method of claim 8 wherein the RNA molecule is introduced by transfection.

11. A method of claims 8-10 wherein the host cell is an animal cell.

12. The method of claim 11 wherein the animal cell is a mammalian cell.

13. The method of claim 12 wherein the mammalian cell is a Chinese Hamster Ovary (CHO) or a Baby Hamster Kidney (BHK) cell.

14. A method of claims 8-13 wherein the animal cell is grown in serum-free medium.

15. The method of claim 14 wherein the serum-free medium is a protein-free medium.

Figure 1:

MTNKCLLQIALLLCFSTTALSMSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDIPEEI
KQLQQFQKEDAALTIYEMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKE
DFTRGKLMSSLHLKRYYGRILHYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN

Figure 2:

aacatgaccaacaagtgtctcctccaaattgctctcctgttgtgcttctccactacagctctttc
catgagctacaacttgcttggattcctacaaagaagcagcaattttcagtgtcagaagctcctgt
ggcaattgaatgggaggcttgaatactgcctcaaggacaggatgaactttgacatccctgaggag
attaagcagctgcagcagttccagaaggaggacgccgcattgaccatctatgagatgctccagaa
catctttgctattttcagacaagattcatctagcactggctggaatgagactattgttgagaacc
tcctggctaatgtctatcatcagataaaccatctgaagacagtcctggaagaaaaactggagaaa
gaagatttcaccaggggaaaactcatgagcagtctgcacctgaaaagatattatgggaggattct
gcattacctgaaggccaaggagtacagtcactgtgcctggaccatagtcagagtggaaatcctaa
ggaacttttacttcattaacagacttacaggttacctccgaaactgaagatctcctagcctgtgc
ctctgggactggacaattgcttcaagcattcttcaaccagcagatgctgtttaagtgactgatgg
ctaatgtactgcatatgaaaggacactagaagattttgaaatttttattaaattatgagttattt
ttatttatttaaattttattttggaaaataaattatttttggtgcaaaagtc

INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/IB 96/01394

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/22 C07K14/565 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	J.VIROL.; 67 (1993), 6439-46, XP000674629 BREDENBEEK, P. ET AL.: "Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs" cited in the application see the whole document ---	1-5,7-13
Y	WO 96 07730 A (RENNER WOLFGANG A ;EPPENBERGER HANS M (CH); BAILEY JAMES EDWIN (CH) 14 March 1996 see the whole document --- -/--	13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 August 1997	26.08.97

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Intern al Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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